Morphine, Oxycodone, Methadone and Its Enantiomers in Different Models of Nociception in the Rat

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We studied the effects of the commonly used μ-opioid receptor agonists morphine, oxycodone, methadone and the enantiomers of methadone in thermal and mechanical models of acute pain and in the spinal nerve ligation model of neuropathic pain in rats. Subcutaneous administration of morphine, oxycodone, and methadone produced a dose-dependent antinociceptive effect in the tail flick, hotplate, and paw pressure tests. *l*-methadone, racemic methadone, and oxycodone had a similar dose-dependent antinociceptive effect, whereas the dose-response curve of morphine was shallower. In the spinal nerve ligation model of neuropathic pain, subcutaneous administration of morphine, oxycodone, methadone and *l*-methadone had antiallodynic effects in tests of mechanical and cold allodynia. *l*-methadone showed the strongest antiallodynic effect of the tested drugs. *d*-methadone was inactive in all tests. Morphine 5.0 mg/kg, oxycodone 2.5 mg/kg, and *l*-methadone 1.25 mg/kg decreased spontaneous locomotion 30 min after drug administration. In conclusion, in acute nociception all μ-opioid receptor agonists produced antinociception, with morphine showing the weakest effect. In nerve injury pain, *l*-methadone showed the greatest antiallodynic potency in both mechanical and cold allodynia compared with the other opioids. Opioids seem to have different profiles in different pain models. *l*-methadone should be studied for neuropathic pain in humans.

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Opioids are the first-line pharmacological treatment for cancer pain. The consumption of opioids has increased significantly since opioids were introduced to the management of chronic noncancer pain. Even though the most commonly used strong opioids such as morphine, oxycodone, and methadone are all μ-opioid receptor agonists (1,2) they have distinctly different molecular structures and may have different effects in different pathophysiological conditions. However, these opioids have not been compared in standardized conditions in different pain models.

Oxycodone is the most commonly used strong opioid in the United States. Most basic research has been performed with morphine, whereas few data are available regarding oxycodone (3). Oxycodone has active metabolites, e.g., oxymorphone, but their role in oxycodone-induced analgesia needs to be clarified (4,5).

Methadone has been suggested to be effective in pain conditions in which other μ-opioid receptor agonists have failed (6) and to have antihyperalgesic or antiallodynic effects that morphine lacks (7–10). However, properly controlled trials have not been performed to test this.

Dextropropoxyphene (11), ketobemidone (12), ketamine (13), and methadone are noncompetitive antagonists of the *N*-methyl-D-aspartate (NMDA)-receptor. The activation of the NMDA-receptor/channel complex at the spinal level and in the brain is related to the activation of the excitatory glutamatergic nociceptive pathway. The activation of the NMDA-system modulates inflammatory and neuropathic pain, opioid-induced analgesia, and tolerance (14–16). The combination of an NMDA-antagonist (MK-801) with morphine has increased the analgesic activity of morphine (17) and blocked morphine-induced conditioned place preference in mice (18).

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Both the *l*- and *d*-enantiomers of methadone have been described to be noncompetitive NMDA-receptor antagonists using the same binding site as MK-801 (19). *l*-methadone is responsible for the μ-opioid effects of racemic methadone, whereas *d*-methadone is only a weak opioid agonist (20).

We studied the antinociceptive and antiallodynic effects of oxycodone and methadone and its enantiomers compared with the standard opioid analgesic morphine. The tail flick, hotplate and paw pressure tests and the spinal nerve ligation (SNL) model of neuropathic pain were used in rats. Sedative effects of these drugs were assessed with spontaneous locomotor activity.

This is the first study comparing, in the same experiment, the antinociceptive and antiallodynic effects of morphine, oxycodone, methadone, and its enantiomers.

Methods

Male Sprague Dawley rats (Taconic Europe, Ry, Denmark), weighing 175–200 g were used. The animals were housed in clear plastic cages with a 12-h artificial light-dark cycle. Water and laboratory chow were available *ad libitum*. The rats were habituated to the testing environment for 30 min/day for 3 days. After the tests the animals were euthanized. The experiments were performed according to the guidelines of local authorities and the International Association for the Study of Pain (21). The institutional animal investigation committee and the provincial government of Southern Finland approved the protocol. The number of animals per group was 7, except in the spontaneous locomotor activity test, in which 5 per group were used. The same animals were used in 3 different tests with a 3 days' interval.

Morphine hydrochloride (2.5–10 mg/kg, 8–31 μmol), oxycodone hydrochloride (1.25–5 mg/kg, 4–14 μmol), and methadone hydrochloride (1.25–5 mg/kg, 4–14 μmol) were purchased from the University Pharmacy, Helsinki, Finland. *d*- and *l*-methadone hydrochloride (1.25–5 mg/kg, 4–14 μmol) were synthesized from methadone hydrochloride. All drugs were dissolved in sterile physiological saline. Physiological saline solution served as a control. All drugs were administered subcutaneously.

The resolution of (±)-methadone was based on the report by Howe and Sletzinger (22) with major modifications described below. (±)-methadone hydrochloride (8.3 g, 24 mmol) was dissolved in 80% ethanol (21 mL) at 35°C, and (–)-ammonium 3-bromocamphor-8-sulfonate (4.1 g, 12.5 mmol) was added to the solution. The resulting mixture was added in portions to crushed ice (185 g) with vigorous stirring, and stored at 0°C for 1 day. The collected crude salt (5.9 g, melting point 127°C–129°C) was dissolved in ethanol and precipitated with ice. The resulting (–)-methadone (–)-3-bromocamphor-8-sulfonate was filtered, washed with small portions of cold water, and dried *in vacuo* (4.8 g, melting point 132°C-134°C, lit. 135°C –138°C).

The filtrate from the preparation of (-)-methadone (–)-3-bromocamphor-8-sulfonate was concentrated to 100 mL *in vacuo*. The concentrate was extracted with ethyl acetate (3 × 50 mL). A 4 M aqueous solution of NaOH (12 mL) was added to the aqueous phase, and the resulting mixture was stirred vigorously and subsequently cooled to 5°C. Stirring was continued until the precipitate was loose and distinctly granular. The mixture was stored at 0°C for 16 h. Precipitates were filtered, washed with ice water (2 × 25 mL), and dried carefully *in vacuo* to give the free methadone base consisting of approximately 75% of (+)-methadone and 25% of (–)-methadone (2.73 g, melting point 76°C, lit. 79°C–81°C) (23). The crude (+)-methadone was dissolved in 1-propanol (5.5 mL) at 70°C, and treated with D-(–)-tartaric acid monopotassium salt (1.66 g, 8.82 mmol). The resulting mixture was stirred at 4°C for 30 min. The precipitated salt was filtered, washed with cold 50% *1*-propanol/*n*-hexane (3 mL) and *n*-hexane (2 × 5 mL), and dried to give the crude salt (melting point 80–81°C). The melting point of the reference (+)-methadone (–)-tartrate was 98°C. Crude salt was re-crystallized from 2-propanol/H_2O using the following method. The resulting salt was dissolved in methanol (150 mL) at reflux; water was added by drops to dissolve the salt completely. The resulting solution was cooled, 2-propanol (20 mL) was added, and methanol was evaporated slowly under reduced pressure. The crystals of (+)-methadone (–)-tartrate formed upon cooling were filtered and dried *in vacuo* (3.23 g, melting point 99°C).

Both (–)-methadone (–)-3-bromocamphor-8-sulfonate and (+)-methadone (–)-tartrate were treated with a 4 M aqueous solution of NaOH (1.5 equivalents) at room temperature to give (–)- and (+)-methadone, respectively. The aqueous phases were extracted twice with ethyl acetate. The organic layers were washed with brine and dried with anhydrous Na_2SO_4. The solutions of (–)- and (+)-methadone in ethyl acetate were concentrated and treated by drops with a 4 M solution of hydrogen chloride in 1,4-dioxane (2 equivalents) under vigorous stirring. The white crystals of (–)- and (+)-methadone hydrochloride were filtered and dried *in vacuo*. (–)-methadone hydrochloride 1.6 g, melting point 241°C, lit. 237°C–239°C (22), $[\alpha]^{25}_D$ = –127 deg · mL · dm^{-1} · g^{-1} (c = 1.0, H_2O). (+)-methadone hydrochloride 0.8 g, melting point 241°C, lit. 243°C–244°C (22), $[\alpha]^{25}_D$ = +127 deg · mL · dm^{-1} · g^{-1} (c = 1.0, H_2O). Additionally, the resolution of (±)-methadone was monitored by means of capillary electrophoresis.

Tail flick latencies were tested with a Ugo Basile (Comerio, Italy) apparatus. The animals were restrained in transparent Plexiglas tubes during the measurement. The tests were performed 3 times (with a 15 s interval) at each time point, and the average of the measurements was used. The cut-off was set at 8 s to avoid tissue damage in the tail.

The hotplate device by Harvard Apparatus Ltd. (Edenbridge, Kent, U.K.) was calibrated to 52.5°C ± 0.3°C and the cut-off time was set at 60 s. Licking or shaking the paw or jumping was considered as a sign of thermal nociception.

The paw pressure test was performed with the Ugo Basile paw pressure device (Comerio, Italy). Each rat was gently wrapped in a towel during the test. The left hindpaw was placed under the weight of the apparatus, and the test was started. A brisk foot withdrawal of the hindleg after constantly increasing pressure terminated the measurement, and the final pressure was recorded.

All 3 nociceptive tests were performed before, and 30, 60, 90, 120, 180, and 240 min after the administration of the drugs or saline.

The ligation of the L5 and L6 spinal nerves was performed as described by Kim and Chung (24) under halothane anesthesia (2% in 50% O_2–50% N_2O). In brief, a small piece of the paravertebral muscle and a part of the left transverse process of the L5 lumbar vertebra were removed to expose the underlying spinal nerves that were gently isolated. A tight 6-0 silk ligature was tied around the L5 and the L6 spinal nerves, and the hemostasis was checked. The muscle was closed with sutures and the skin with metal clips. The behavioral tests were performed 2 wk after surgery. During the recovery, the condition of the animals was checked daily and animals with any neurological symptoms not typical to the model (paralysis of the operated limb, difficulties in walking) were immediately killed ($n = 3$).

In the SNL animals von Frey filaments were used to determine the threshold for mechanical allodynia. Rats were placed on a metal mesh covered with individual transparent plastic cages, which enabled the observation of the behavior of the animals and stimulation of the plantar surface of the ipsilateral paw with a series of von Frey filaments. Testing started with the strongest filament used (12.5 g) and continued to the next lighter filament until the lightest filament producing a brisk foot withdrawal or lifting and licking of the paw in more than half of the stimulations was found. To avoid excessive stimulation during the drug tests, the probing was started in the following testing sessions with the weakest hair that had elicited withdrawal responses in the previous session. If the strongest hair (12.5 g) did not produce a response, 12.5 g was recorded as the threshold.

To test cold allodynia, a drop of acetone was applied to the plantar surface of the ipsilateral paw with a syringe connected to a thin polyethylene tube (25). The number of paw withdrawals out of three consecutive applications was recorded. These 2 tests were performed in this order before and 30, 60, 90, 120, 180, and 240 min after the administration of the drugs or saline.

Possible sedative effects of the opioids were measured as reduction of spontaneous motor activity in a closed dark field isolated to sound (70 × 70 × 35 cm) where the rats were placed one at a time. Photocells were located at 2 different levels inside the box (2 cm and 12 cm above the cage floor) to detect movements of the animal. A 30-min measurement was started 30 min after the drug injection ($n = 5$ for each drug). As nearly all of the motor activity was concentrated to the first 15 min, this period was used for statistical analysis.

The results of the nociceptive tests where a cut-off value was used to prevent tissue damage (the tail flick test, the hotplate test and the paw pressure test) are shown as mean of the maximum percentage effect, calculated as: MPE% = (postvalue − prevalue)/(cut-off − prevalue) × 100%. Because of the semiquantitative nature of the allodynia tests (von Frey hairs and the acetone drop cold allodynia test), the area under the effect-time curve was used, expressed as percentage of the full effect (AUC%). To allow assessment of the data quality, examples of the mean raw values are shown for selected doses. Nonparametric analysis of variance (the Kruskal-Wallis test) with StatView 5.5 (SAS Institute Inc., Cary, NC) was used for statistical analysis.

Results

Morphine, oxycodone, methadone, and *l*-methadone all produced dose-related antinociception in the tail flick, hotplate, and paw pressure tests (Figs. 1, 2 and 3). The maximum effect was observed at 30 min after subcutaneous drug administration. The dose-response curve of morphine was significantly shallower compared with those of oxycodone and methadone particularly in the hotplate test. Oxycodone and *l*-methadone induced a rapid antinociceptive effect with a shorter duration compared with morphine and methadone. In the tail flick test the smallest drug doses that produced full 100% MPE were oxycodone 2.5 mg/kg, *l*-methadone 2.5 mg/kg, methadone 5.0 mg/kg, and morphine 10.0 mg/kg. In the hotplate test *l*-methadone was most effective and a 100% MPE was achieved with 2.5 mg/kg whereas the required doses for oxycodone and morphine were >5.0 mg/kg and >10 mg/kg, respectively. In the paw pressure test a 100% MPE was achieved with 5.0 mg/kg of oxycodone and *l*-methadone and 10 mg/kg of morphine.

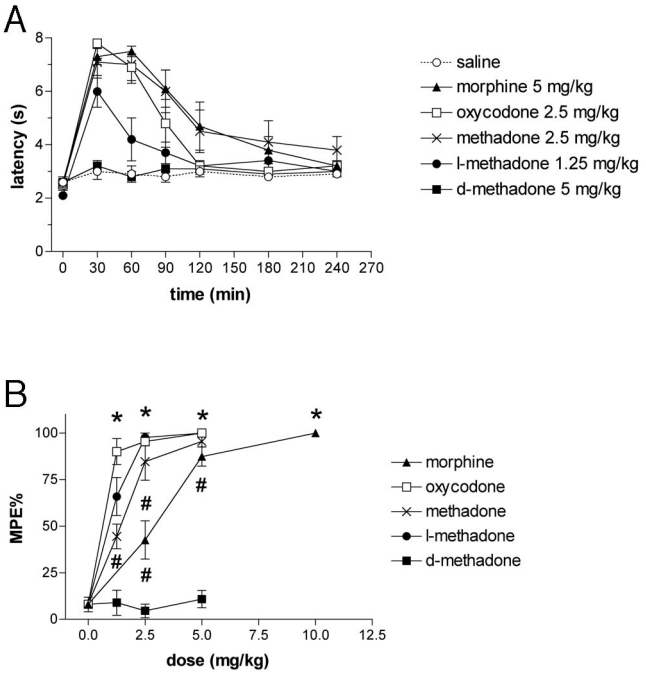

Figure 1. Opioid effects of the tail flick test in rats. A, Time-course of the antinociceptive effect of selected doses of the opioids studied. Tail flick latencies (s ± SEM) after subcutaneous administration of morphine (5 mg/kg), oxycodone (2.5 mg/kg), methadone (2.5 mg/kg), *l*-methadone (1.25 mg/kg), and *d*-methadone (5 mg/kg) are plotted over time after drug administration (min). $n = 7$ animals per group. B, Dose-response curves for each study drug in the tail flick test in rats. The mean of the maximum possible effect (MPE%) ± SEM at 30 min is shown for each dose. The symbols indicate statistically significant differences ($P < 0.05$) as compared with the saline control (*), and among the study drugs (#). $n = 7$ animals per group.

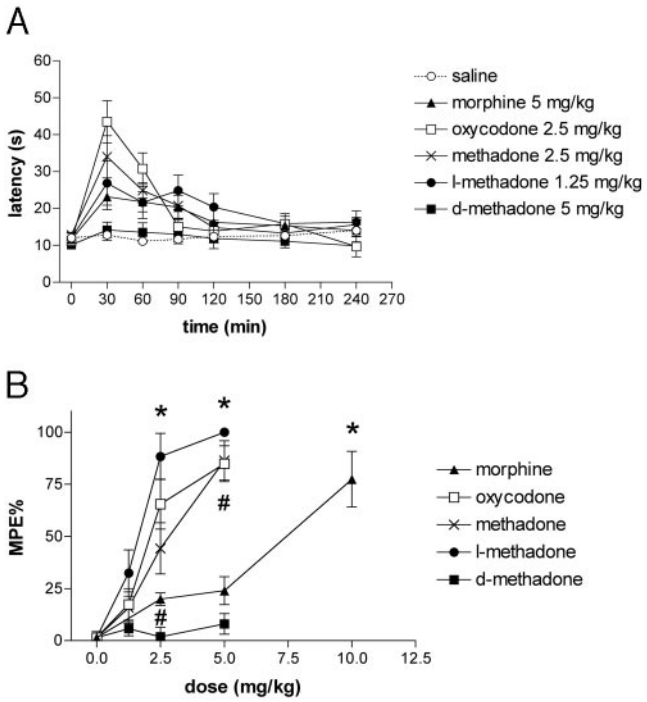

Figure 2. Opioid effects in the hotplate test in rats. A, Time-course of the antinociceptive effect of selected doses of the opioids studied. Hotplate latencies in (s ± SEM) after subcutaneous administration of morphine (5 mg/kg), oxycodone (2.5 mg/kg), methadone (2.5 mg/kg), *l*-methadone (1.25 mg/kg) and *d*-methadone (5 mg/kg) are plotted over time (min). $n = 7$ animals per group. B, Dose-response curves for each study drug. The mean of the maximum possible effect (MPE%) ± SEM at 30 min is shown for each dose. The symbols indicate statistically significant differences ($P < 0.05$) as compared with the saline control (*), and among the study drugs (#). $n = 7$ animals per group.

d-methadone and saline did not show any antinociceptive effects in any of the antinociceptive tests.

In the von Frey filament test for mechanical allodynia morphine, oxycodone, methadone, and *l*-methadone all produced significant, dose-related antiallodynic effects (Fig. 4). Morphine 5 mg/kg, oxycodone 1.25, 2.5 mg/kg, methadone 1.25, 2.5 mg/kg, and *l*-methadone 1.25 mg/kg had significant antiallodynic effects compared with saline. *l*-methadone was most potent against mechanical allodynia (Fig. 4b). *d*-methadone (1.25–5.0 mg/kg) and saline had no antiallodynic effect in this test.

In the acetone test for cold allodynia morphine, oxycodone, methadone, and *l*-methadone all produced a dose-related antiallodynic effect with *l*-methadone showing the greatest potency (Fig. 5). *d*-methadone (1.25–5.0 mg/kg) and saline had no effect on the cold sensitivity in this test.

Muscle rigidity was observed after morphine 10 mg/kg, oxycodone 5 mg/kg, methadone 5 mg/kg and *l*-methadone 2.5–5 mg/kg administration. Degree of muscle rigidity was not graded.

In the spontaneous locomotor activity test the rats were tested with the antiallodynic doses of morphine (5 mg/kg), oxycodone (2.5 mg/kg), and *l*-methadone (1.25 mg/kg). Subcutaneous administration of oxycodone 2.5 mg/kg, morphine 5.0 mg/kg, and l-methadone 1.25 mg/kg caused statistically significant reduction of spontaneous locomotor activity compared with saline (Fig. 6). The largest reduction of spontaneous locomotor activity was observed after oxycodone 2.5 mg/kg administration.

Discussion

In the present study oxycodone was more potent in all nociceptive tests compared with morphine. The difference between oxycodone and morphine is interesting as the μ-opioid receptor binding potential of oxycodone is significantly less than that of morphine (26). A similar difference in antinociceptive potency between oxycodone and morphine in acute nociception has also been shown in female Wistar rats (27). A recent study indicated that oxycodone was >10 fold less effective than morphine in the [^{35}S]GTPγS-binding assay that measures the G-protein-mediated activation of the μ-opioid receptor by the agonist (28).

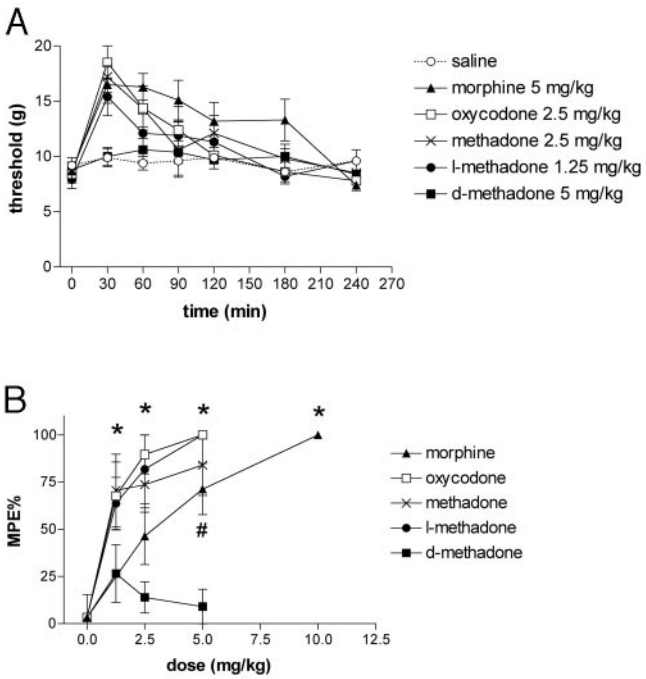

Figure 3. Opioid effects in the paw pressure test in rats. A, Time-course of the antinociceptive effects of selected doses of the opioids studied. Mean paw pressure thresholds (g) ± SEM after subcutaneous administration of morphine (5 mg/kg), oxycodone (2.5 mg/kg), methadone (2.5 mg/kg), *l*-methadone (1.25 mg/kg), and *d*-methadone (5 mg/kg) are plotted over time (min). $n = 7$ animals per group. B, Dose-response curves for each study drug. The mean of the maximum possible effect (MPE%) ± SEM at 30 min is shown for each dose. The symbols indicate statistically significant differences ($P < 0.05$) as compared with the saline control (*), and among the study drugs (#). $n = 7$ animals per group.

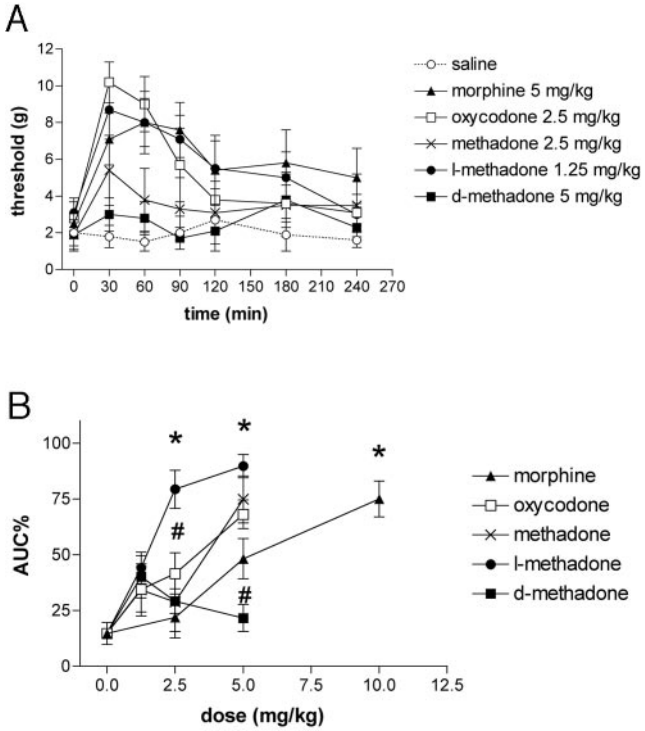

Figure 4. Opioid effects on mechanical allodynia in rats with the spinal nerve ligation model of neuropathy. A, Time course of the antiallodynic effect of selected doses of the opioids studied. The mean force that induced paw withdrawal response (g) ± SEM after subcutaneous administration of morphine (5 mg/kg), oxycodone (2.5 mg/kg), methadone (2.5 mg/kg), *l*-methadone (1.25 mg/kg), and *d*-methadone (5 mg/kg) as measured with the von Frey hairs is plotted over time (min). $n = 7$ animals per group. B, Dose-response curves for each study drug in the von Frey hair test. The attenuation of mechanical allodynia is shown as the area under the curve (AUC%) ± SEM. The symbols indicate statistically significant differences ($P < 0.05$) as compared with the saline control (*), and among the study drugs (#). $n = 7$ animals per group.

It remains to be seen whether the fast and effective antinociception produced by systemic oxycodone is attributable to its potent metabolites, e.g., oxymorphone.

Also, *l*-methadone was significantly more potent in acute nociception compared with morphine. The difference was particularly obvious in the hotplate test. Methadone shows stronger μ-opioid receptor binding than morphine and it is also significantly more lipophilic (29).

In this study both thermal and mechanical tests of acute nociception were used. Hotplate and paw pressure tests require supraspinal processing of the nociceptive information and differ from the tail flick test, which is primarily a spinal reflex. Results of the thermal tests show significant reduction in the potency of morphine in the hotplate test (Fig. 2b) compared with the tail flick test (Fig. 1b), whereas no significant differences between morphine and other active drugs studied were observed in the paw pressure test. The mechanism behind this discovery remains unknown.

Oxycodone, methadone, and particularly *l*-methadone showed significant antiallodynic effects against both mechanical and cold allodynia in the SNL model of neuropathic pain. Morphine showed antiallodynic effects at larger doses only. At the antiallodynic doses morphine, oxycodone and *l*-methadone decreased spontaneous locomotor activity that is considered to indicate sedation. This is an expected effect in opioid-naïve rats and humans. This may interfere with the stimulus discrimination, particularly if the stimulus is not strong. The reduction of spontaneous locomotor activity by the doses used did not produce cataleptic-like behavior but was graded as a sign of sedation with this sensitive variable. Only the largest dose of *l*-methadone (5 mg/kg) produced spastic muscle rigidity, which was not graded. The results on the antiallodynic effects agree with the clinical evidence of opioids being effective for neuropathic pain. Oxycodone relieves neuropathic pain in postherpetic neuralgia (30) and diabetic polyneuropathy (31). Also, morphine and methadone have been shown to be effective in postherpetic neuralgia (32). No direct head-to-head comparisons among these three opioids

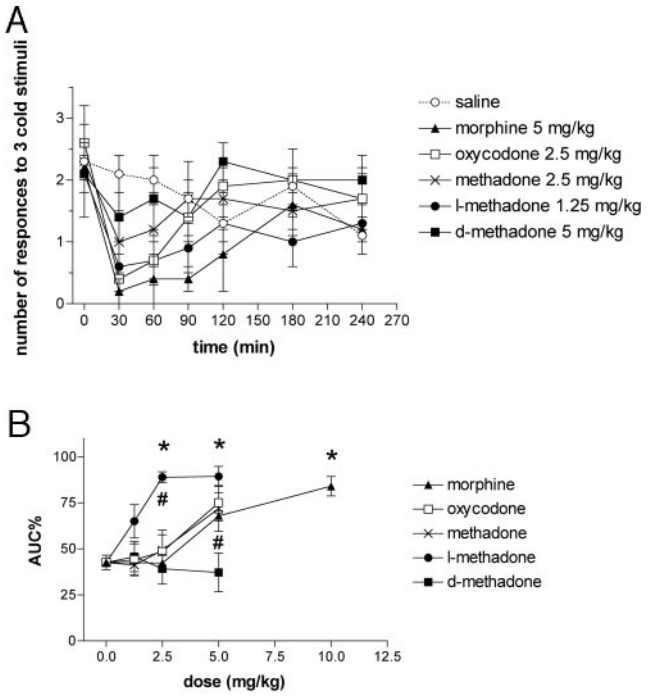

Figure 5. Opioid effects on cold allodynia in the rats with spinal nerve ligation model of neuropathy. A, Time-course of the antiallodynic effects of selected doses of the opioids studied. The mean number of paw withdrawal responses to 3 applications of acetone (± SEM) after subcutaneous administration of morphine (5 mg/kg), oxycodone (2.5 mg/kg), methadone (2.5 mg/kg), *l*-methadone (1.25 mg/kg), and *d*-methadone (5 mg/kg) is plotted over time. $n = 7$ animals per group. B, Dose-response curves for the opioid effects in the acetone test for cold allodynia. The attenuation of cold allodynia is shown as the area under the curve (AUC%) ± SEM. The symbols indicate statistically significant differences ($P < 0.05$) as compared with the saline control (*), and among the study drugs (#). $n = 7$ animals per group.

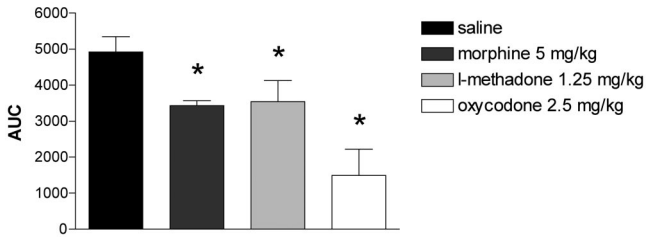

Figure 6. Opioid effects on motor activity in rats. Effects of morphine (5 mg/kg), *l*-methadone (1.25 mg/kg) and oxycodone (2.5 mg/kg) on spontaneous locomotion 30–45 min after subcutaneous administration of the opioids. The area under the curve (AUC) of the activity counts (number of photocell crossings ± SEM) over time is given for each study drug. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) compared with the saline control group. $n = 5$ animals per group.

have been made, however. The present results indicate that *l*-methadone would be a particularly interesting drug to be assessed for clinical neuropathic pain.

Glutamatergic activation is related to inflammatory and neuropathic pain. The reports that methadone can be effective in some pain conditions that do not respond to morphine or other μ-opioid receptor agonists have led to the speculation that racemic methadone has NMDA-receptor antagonist activity *in vivo*. Both enantiomers of methadone have been shown to act as noncompetitive antagonists of the NMDA-receptor *in vitro* (19). If this were true, the *d*-enantiomer of methadone should increase the analgesic activity of other μ-opioid agonists like morphine. *d*-methadone has been shown to produce antinociception in formalin-induced inflammation in rats. The effect was not reversed by naloxone (33), indicating μ-opioid receptor-independent activity. In the same study *d*-methadone was shown to be inactive in the tail flick test in agreement with the present results. IV *d*-methadone was found to be active against mechanical hyperalgesia after peripheral inflammation, and it inhibited the responses of hindlimb single motor units to noxious electrical and mechanical stimuli (34). These actions were abolished with pretreatment with naloxone, indicating an opioid action. It was concluded that even though *d*-methadone shows affinity to the NMDA-receptor channel *in vitro*, the NMDA-antagonism does not appear to contribute to the antinociceptive action of systemic *d*-methadone *in vivo*. *d*-methadone did not enhance the antinociceptive potency of morphine in the tail flick test in mice (35). However, *d*-methadone has been found to attenuate NMDA-induced hyperalgesia in rats (36).

In the present study *d*-methadone was inactive in all tests of nociception and neuropathy at the doses studied. A previous *in vivo* electrophysiological study with anesthetized rats indicated that the neuronal inhibitory effects of racemic methadone and large doses of *d*-methadone are naloxone reversible, indicating an opioid mechanism of action (37). In an earlier binding study *l*-methadone had a 10-fold higher affinity for mu1 receptors than *d*-methadone (IC50 3.0 nM and 26.4 nM, respectively). At the mu2 receptor, the IC50 value of *l*-methadone was 6.9 nM and 88 nM for *d*-methadone, respectively. As expected, *l*-methadone had twice the affinity for mu1 and mu2 receptors than the racemate. These result suggest that *d*-methadone does not essentially contribute to opioid effect of racemic methadone (1). *l*-methadone was at least as effective as racemic methadone at half the dose. The opioid activity of racemic methadone in the present study thus seems to be mediated through *l*-methadone only.

Our results show that systemic morphine, oxycodone, methadone, and *l*-methadone all produce dose-dependent antinociception in models of acute thermal and mechanical nociception. Morphine was the least potent of the opioids tested. In the model of chronic neuropathic pain, the antiallodynic doses of all opioids caused central nervous system depression. *d*-methadone was inactive in all tests, indicating that

l-methadone is responsible for the analgesic effects of racemic methadone. These results support the clinical impression that methadone is an effective opioid for neuropathic pain. Controlled clinical trials are needed to confirm this.

The development of tolerance and opioid-induced hyperalgesia are important clinical problems. Whether oxycodone and particularly methadone show any advantages over morphine in these conditions should also be studied.

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